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Review

Rhomboid proteases in mitochondria and plastids: Keeping organelles in shape ☆

Danny V. Jeyaraju [a], Aditi Sood [a], Audrey Laforce-Lavoie [a], Luca Pellegrini [a,b,*]

[a] Institut universitaire en santé mentale de Québec, Université Laval, Québec, Canada
[b] Department of Molecular Biology, Medical Biochemistry and Pathology, Faculty of Medicine, Université Laval, Québec, Canada

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ABSTRACT

Rhomboids constitute the most widespread and conserved family of intramembrane cleaving proteases. They are key regulators of critical cellular processes in bacteria and animals, and are poised to play an equally important role also in plants. Among eukaryotes, a distinct subfamily of rhomboids, prototyped by the mammalian mitochondrial protein Parl, ensures the maintenance of the structural and functional integrity of mitochondria and plastids. Here, we discuss the studies that in the past decade have unveiled the role, regulation, and structure of this unique group of rhomboid proteases. This article is part of a Special Issue entitled: Protein Import and Quality Control in Mitochondria and Plastids.

1. Introduction

Proteases are enzymes that catalyze the hydrolysis of the peptide bond that links amino acids together in a protein; they participate in proteins catabolism and act as regulators of nearly every biological process [1]. Proteases are grouped in different catalytic types, depending on the catalytic amino acid residue used to conduct the nucleophilic attack on the peptide-bond of the substrate: aspartic, cysteine, glutamic, metallo (His), asparagine, threonine, and serine proteases. Due to the requirement of water for the proteolytic reaction, proteases were generally thought to hydrolyze their substrates in an aqueous environment. However, in 1997 Rawson and colleagues reported the discovery of site-2 protease, the first kind of protease capable of executing hydrolysis within the water-excluding environment of the phospholipid bilayer, which composes most of the biological membranes [2]. Such unique type of proteases are termed intramembrane-cleaving proteases, or I-CLiPs, and are structurally characterized by having the catalytic site residues projecting in a cavity that is buried within the lipid bilayer of the membrane in which the I-CliP is embedded [3].

To date, most of the I-CliP substrates appear to be membrane proteins; their cleavage typically occurs at or near the transmembrane helix (TMH) that anchors the substrate to the lipid bilayer [4,5]. Thus, their proteolysis serves to either complete protein maturation, or to release membrane-tethered domains, which are typically used for signaling [6]. Over the past decade, these released moieties have been shown to engage in intra- and inter-cellular communication, mainly by acting as transcription factors or ligands of signaling factors in a process that is termed regulated intramembrane proteolysis [7,8].

There are four known families of I-CliPs: site-2 protease, first discovered as a regulator of cholesterol biosynthesis [2,9]; presenilins/gamma secretase, implicated in Notch signaling [10] and Alzheimer's disease [11,12]; signal peptide peptidase [13,14]; and the rhomboids [15].

2. Rhomboids genes

The Rhomboid gene was first discovered in Drosophila. Fly developmental studies classified it under the Spitz group of genes, which includes *Spitz*, *Star*, *Pointed*, *Rhomboid*, *Single-minded*, and *Sichel*: mutations in any of these cause similar pattern alterations in ventral ectodermal derivatives of the fly embryo [16]. Because the names of these genes described the phenotype of the mutant larval cuticle, the mis-shaped, rhombus-like head skeleton of the mutant embryo earned to the *Rhomboid-1* gene its name.

Rhomboid genes are present in most of the sequenced bacterial, archaeal and eukaryotic genomes [17]. Most of the bacterial and archaeal genomes have a single rhomboid gene. In contrast, eukaryotes show expansion of the rhomboid family, with 2 members in yeast, 7 in Drosophila, 5 in humans and as many as 13 in *Arabidopsis t.* [18]. Phylogenetic analysis revealed that eukaryotic rhomboids are split between two

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* Corresponding author at: Institut universitaire en santé mentale de Québec, Université Laval, Québec, Canada.
E-mail address: luca.pellegrini@crulrg.ulaval.ca (L. Pellegrini).

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major subfamilies, which are positioned in the midst of different prokaryotic branches [17]. The first subfamily was designated the RHO subfamily [17]; its prototype is the Drosophila developmental regulator Rho-1 [15,19–21]. The second eukaryotic subfamily was designated the PARL subfamily, and its prototype is the mammalian Parl protein which encodes for a mitochondrial inner membrane protein (discussed below) [17]. Whereas in plants the PARL family is considerably more represented, in animals there has been a selective expansion only of RHO family members; indeed, animal genomes typically contain only one PARL gene [17]. What evolutionary constraints have led, in animals, to the selective expansion of the RHO subfamily and, possibly, to the selected elimination of additional members of the PARL subfamily, remain unknown. Instead, the remarkable expansion of the PARL subfamily in plants appears to be linked to the presence of plastids: major plant- and algae-specific self-replicating organelles that are the site of photosynthetic fixation of CO_2 and are responsible for the exclusive manufacture and storage of starch and lipids. Although plastids greatly differ by their pigmentation, function, and ultrastructure, they are thought to have originated from endosymbiotic cyanobacteria [22]: such symbiosis parallels the event that lead to the acquisition of mitochondria in eukaryotes, which required the acquisition of a PARL family member to control inner membrane remodeling [18]. Thus, the expansion of the PARL family in plants could reflect the need to coordinate the remodeling and organization of the chloroplast thylakoid membrane, whose structure and dynamics is more complex than that of the mitochondrial *cristae*.

Phylogenetic analysis also identified a subgroup of rhomboid genes that express proteins lacking the catalytic residues: pseudoproteases [17]. These genes, which are present in all sequenced metazoans and are present only in metazoans, are termed iRhoms, for inactive rhomboids [23]. Unlike most sequenced metazoans, Drosophila has only a single iRhom gene, *rhomboid-5*. The high degree of conservation of iRhoms genes indicates that these pseudoproteases have been under evolutionary selection pressure, which in turn indicates that they possess a relevant biological function. Recent studies have indeed shown a critical role of iRhoms in regulating growth factor signaling through endoplasmic reticulum-associated protein degradation (ERAD) [24], and in the innate immunity response [25,26]. Derlin-1, a prominent component of the ERAD pathway [27], and iRhom2 [25,26] are the most recently characterized members of the iRhom group of pseudoproteases in mammals.

3. Rhomboids evolutionary origin

Phylogenetic analysis indicates that rhomboid family emerged in some bacterial lineage and widely disseminated by horizontal gene transfer (HGT) [17]. Both archaea and eukaryotes seem to have acquired rhomboids on several independent occasions. In particular, at least two HGT events seem to have contributed to the origin of eukaryotic rhomboids, one of them yielding the RHO subfamily and the other one the PARL subfamily, with a possible additional HGT in plants [17].

4. Rhomboids structure

Rhomboids are intramembrane-cleaving serine proteases. They are among the most conserved family of polytopic membrane proteins known to date and the most ancient type of I-CliP [17]. Prokaryotic and eukaryotic rhomboid proteins share only 10–15% of sequence identity in their catalytic domain (Fig. 1A); this is not surprising since polytopic transmembrane proteins are, in general, not strongly conserved. Rhomboid proteases from all kingdoms of life share the fact of having the catalytic domain composed by six TMHs [17]. Phenomenal work in protein crystallography has determined the structure of this domain in bacterial rhomboids: the overall shape is a compact helical bundle comprised of six TMHs and a lateral protrusion that connects TMH-1 and -2 [28–33]. Nearly the entire rhomboid domain appears to be immersed in the phospholipid bilayer; the lateral protrusion is only partially immersed in the membrane and might, therefore, serve to properly position the protease [33–35]. The universally conserved catalytic Ser residue, on TMH-4, lies submerged ~10 Å from the presumed plane of the membrane, making the site of catalysis intramembrane.

The structures of the bacterial rhomboids have revealed that the catalytic Ser and His residues in TMH-4 and TMH-6, respectively, are spatially arranged in a manner akin to classical serine proteases. In dyad-based serine proteases, the serine–histidine pair act together to generate a nucleophile of sufficient power to attack the carbonyl group of a peptide bond [36]. However, several serine proteases use a triad-based mechanism of catalysis [37]; here, a third catalytic residue, typically Asp or Asn, plays an important, but not essential, role in stabilizing the tetrahedral intermediate and the transition state leading to it. The structures of the bacterial rhomboids have unequivocally shown the existence of a catalytic dyad; indeed, a third conserved Asn residue, which was suspected to act as a third catalytic residue, is too remote and geometrically not in position to contact the catalytic His and form a triad [28–33]. However, recent studies form our laboratory have shown that eukaryotic members of the PARL family of mitochondrial rhomboid proteases, but not of the RHO family, could use a strongly conserved Asp residue on TMH-5 (Fig. 1D) in a triad-based mechanism of catalysis (Fig. 1C) [38]. The reason why a mitochondrial rhomboid would require a catalytic triad to properly work could be linked to the unique biophysical environment of the organelle. Bacterial rhomboid activity is highly sensitive to the phospholipid composition of the membrane in which they are embedded. Accordingly, the unique lipid composition of the inner mitochondrial membrane, along with the large electrochemical and pH gradient present inside the organelle, might have applied a selective evolutionary pressure that, ultimately, generated a form of the rhomboid protease with a more efficient mechanism of catalysis.

Unlike bacterial rhomboids, eukaryotic members of the RHO and PARL families possess seven TMHs; of these, 6 TMHs constitute the catalytic rhomboid domain. However, major structural differences differentiate these two types of rhomboid proteases. RHO family members, which are localized in the Golgi and plasma membrane, have the 7th TMH added at the carboxy-terminus of the 6-TMH rhomboid core; this topology is referred to as the "6 + 1" structure. Instead, the domain architecture of the rhomboids of the PARL subfamily, which in animals are exclusively found in the mitochondrion and in plants are likely localized in the membranes of the plastids, is characterized by having the 7th TMH added to the amino-terminus of the 6-TMH rhomboid domain; this topology constitutes the "1 + 6" structure [17,18]. The function of the seventh TMH, termed TMH-A and TMH-B in PARL and RHO family members, respectively (Fig. 2A), has not been established for any member of the PARL or RHO family. However, it may play a role in determining the correct topology and orientation of the protein in the membrane, in substrates gating or, in PARL family members, to orient TMH-5 in a way that allows the protease to use a catalytic triad [18,38] (Fig. 1C).

Structure–function studies of the mammalian Parl rhomboid domain shows a remarkable structural conservation of this fold with that of the bacterial GlpG protein (Fig. 1B). However, differences seem to exist. As mentioned above, our studies suggest that an Asp residue on the TMH-5 of Parl (D_{319}; Fig. 1D), which is not present in bacterial and RHO rhomboids, could play a critical structural and catalytic role. Indeed, replacing D_{319} with Asn, Glu, Ala, or Leu profoundly disrupted Parl expression and activity. In bacteria, TMH-5 is a highly dynamic helix [30,34]: a similar property in Parl could allow D_{319} to be positioned in close proximity, and on the same plane, of the catalytic H_{335} residue (Fig. 1C), thereby conferring to Parl a triad-based mechanism of catalysis. This is a likely possibility: mutations designed to assess whether TMH-5 may rotate to bring D_{319} into the active site (Fig. 1C) support indeed this scenario [38].

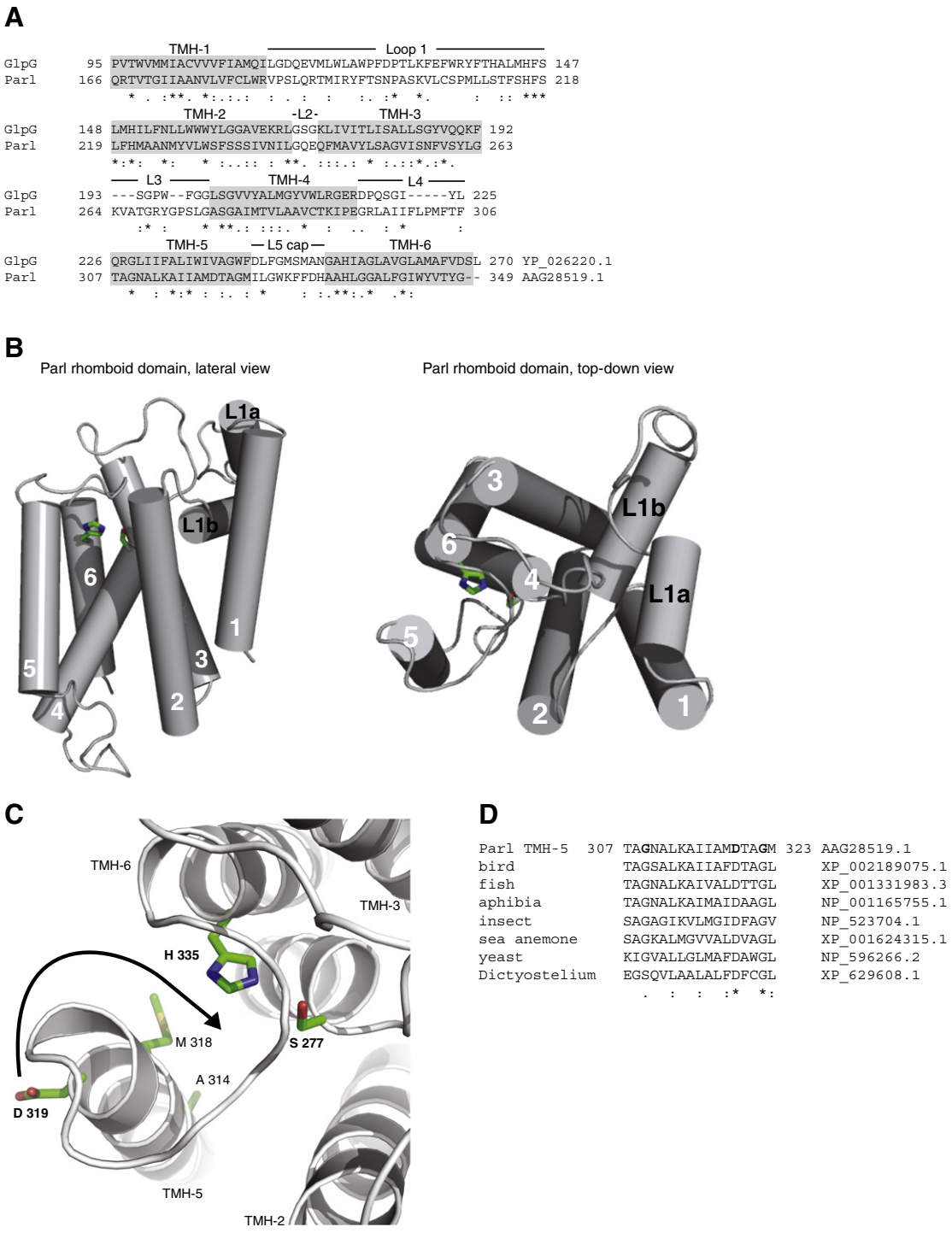
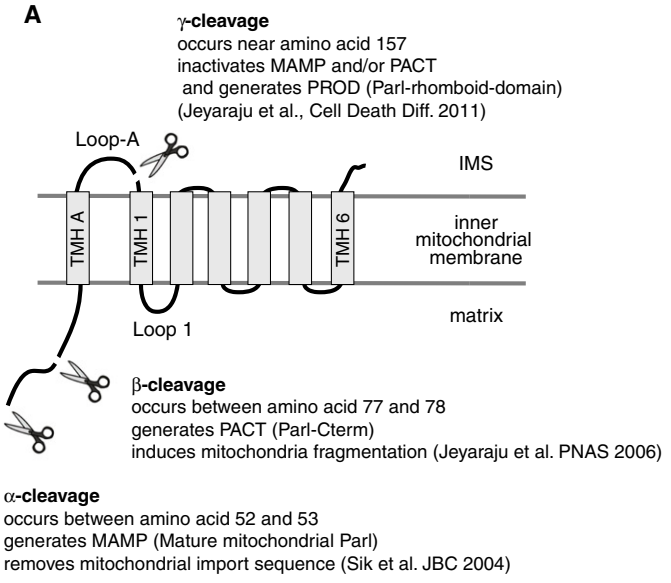


Fig. 1. (A) Sequence alignment of GlpG and Parl used to build homology models. GlpG is a bacterial rhomboid of known structure that served as a template for homology modeling of Parl rhomboid domain (PROD). Transmembrane helices (TMHs) are shaded in gray. (B) Overall topology of PROD with TMHs depicted as cylinders. The catalytic S277 and H335 residues lie on TMH-4 and -6, respectively (depicted as sticks in this figure). (C) The PARL-specific D319 is critical for Parl expression and activity, and might serve as a catalytic residue. In PROD models, D319 is pointed into the membrane bilayer. Note that a rotation of TMH-5 would bring this residue adjacent to H335, where it could serve as a third catalytic residue. (D) Strict conservation of D319 on the TMH-5 of eukaryotic orthologs of Parl. Note that this residue is conserved only in PARL family members and not in RHO-family members or in bacterial rhomboids.
Figures were reproduced from [38].

Noteworthy, the predicted "flexibility" of Parl TMH-5 is consistent with the extensive plasticity of the IMM as well as with the notion that mitochondria *cristae* junctions depend upon Parl activity for their assembly [39–41]. Thus, the bilayer composition and fluidity within and around *cristae* junctions could play a central role in the intrinsic regulation of Parl catalytic activity through the dynamic movement of TMH-5, consistent with findings that indicate the importance of lipid composition in bacterial rhomboid activity regulation [42–44].

iRhoms are also rhomboids, but they lack protease activity due to the absence of key residues and/or domains. They reside in the ER and are considerably larger than active rhomboids, with very long N-

terminal domains preceding the membrane-integral domain and an expanded and conserved L1 loop between TMH-1 and TMH-2 (240–270 amino acids compared with 32 amino acids for the bacterial GlpG protein) [45]. However, exceptions to such topological organization exist (*e.g.* Derlin-1). Given the increasingly important role that iRhoms play in cell biology and disease [25,26,46], their crystallographic analysis will provide major insights on the structural determinants that differentiate the activity of these pseudoproteases from that of true rhomboid proteases.



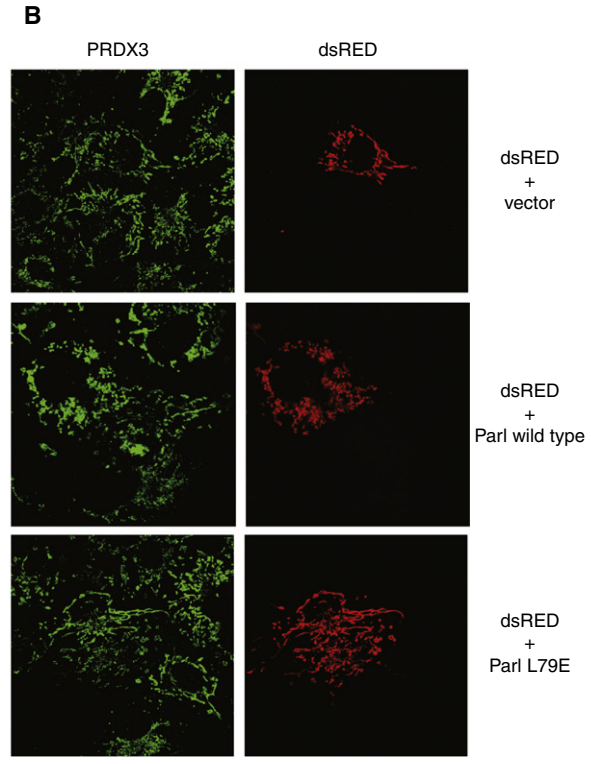


Fig. 2. (A) Scheme showing the proteolytic processing of Parl that generates three forms of the enzyme: MAMP, PACT and PROD. (B) β-cleavage of Parl induces mitochondrial fragmentation. Note that cells transfected with wild type Parl have fragmented mitochondria whereas cells transfected with β-cleavage resistant Parl mutant L79E have elongated, rod like mitochondria.
Figures were reproduced from [38].

5. Mitochondrial rhomboids

Rhomboid proteases have been ascribed to regulate a broad range of biological processes: initiating cell signaling in animals [20], facilitating bacterial quorum sensing in prokaryotes [47,48], dismantling adhesion complexes of parasitic protozoa [49–51], regulate plastid proteins translocation [52], and regulating mitochondrial homeostasis [53,54]. Here, we will focus on the role of mitochondrial rhomboid in regulating the biology of the organelle and on its rising impact on human diseases.

6. Pcp1/Rbd1, the yeast mitochondrial rhomboid

The yeast *Saccharomyces cerevisiae* has two rhomboid genes: *Rbd1* and *Rbd2*. Of these, *Rbd1* encodes a mitochondrial rhomboid protease that, being associated to the processing of the cytochrome c peroxidase protein (Ccp1p), was named Pcp1 (Uniprot P53259) [55]. *Pcp1/Rbd1* codes for a polypeptide of 346 amino acid residues (38.8 kDa) that contains a MitoProt-predicted mitochondrial targeting sequence (score: 0.99). Consistent with the 1 + 6 structure that defines every PARL family member, the protein has a predicted TMH ($_{106}$PLGSMTILGLSLMAGIYFG$_{124}$) appended at the N-terminus of its 6-TMH rhomboid domain, which spans amino acids 143–331. The protease is inserted in the inner mitochondrial membrane, where it serves to cleave Ccp1p [55] and, perhaps more importantly, the dynamin-related protein Mgm1 [53,56], a dynamin GTPase implicated in mitochondria fusion. Additional substrates of Pcp1/Rbd1 might exist, but their identification is hindered by the fact that the phenotype of yeast lacking Pcp1/Rbd1 activity is heavily impacted by the loss of Mgm1 processing. Genetic ablation of *Pcp1/Rbd1* has a profound effect on mitochondrial shape, which becomes fragmented [53]: this phenotype is similar to that of yeast strains lacking other regulators of mitochondrial fusion [57–60]. However, although it is clear that the mitochondrial rhomboid protease in yeast has a critical role in regulating mitochondria fusion and *cristae* morphology through the cleavage of Mgm1 [61], in *Pcp1/Rbd1*−/− cells mitochondrial fusion *per se* is not ablated [62]. As a result of lacking Mgm1 cleavage, however, *Pcp1/Rbd1*−/− *yeast* cells cannot grow on non-fermentable medium and have compromised ability to maintain their mitochondrial DNA content [53,62,63]. Importantly, this phenotype can be restored by expressing the mammalian ortholog of *Pcp1/Rbd1*, *Parl* [53,63].

7. Rho-7, the fly mitochondrial rhomboid

The protein encoded by the Drosophila *Rhomboid-7* gene (*Rho-7*) is a 351 amino acid long mitochondrial rhomboid protease with the typical 1 + 6 structure of the PARL family (Uniprot A1Z8R8). Deletion of *Rho-7* transcriptional start site and of the first 18 codons, which encode part of the predicted mitochondrial import peptide of the protein (Mitoprot score: 0.99), causes 90% of the flies to die before pupariation; however, most of the flies that escape death die within three days, during the process of emerging from the pupal case [64]. Few male and female flies live, but the males are sterile. Further, the surviving mutant flies display a wing-position defect: in these flies, the wings hang down on either side of the abdomen, instead of being positioned on top of the abdomen. As a result, these flies are unable to fly, have extreme difficulty walking, and display erratic twitching in their legs and head, which correlate to severe synaptic defects. Contrary to what is observed in mammals [40], ablation of the mitochondrial rhomboid gene in Drosophila does not sensitize cells to death-inducing insults [64].

Overexpression of the Rho-7 protein rescues the lethality observed in *Rho-7*−/− mutants, but in wild-type flies ectopic overexpression of the protease is semi-lethal (86% die) [65]. The selective over-expression of Rho-7 in the nervous system is also semilethal, albeit to a reduced extent (49%); under this condition, the surviving flies develop into adults but have a shortened life span. Also, nearly 30% of larvae with this genotype display loss of motor coordination; the remaining moved significantly less coordinated compared to control larvae. This phenotype is accompanied by severe mitochondria aggregation, lower ATP levels (42% compared to wild-type flies), and an overall decreased number of organelles within nerves/axons [65]. Consistent with these findings, and with the data from yeast, silencing of *Rho-7* in Drosophila S2 cells results in fragmented mitochondrial morphology. Further, electron microscopic examination of myofibrils of wings in flies lacking Rho-7 activity shows mitochondria of reduced size [64]. Together with the genetic interaction observed between *Rho-7* and *Opa1-like* fusion [64]. However, it should be noted that the structure of the *cristae* in *Rho-7*−/− cells is far less compromised than that observed in *Pcp1/Rbd1*−/− yeast cells; also, that the phenotype of flies lacking *bona fide* mitochondria fusion proteins does not resemble that of *Rho-7*−/− flies. Thus, further investigations are required to firmly establish a role of the drosophila mitochondrial rhomboid protease Rho-7 in mitochondrial membranes fusion.

Pioneering studies have made emerge a role of Rho-7 in mitochondria quality control. *Rho-7*−/− flies exhibit remarkable phenotypic similarity with *PINK1*−/− and *Parkin*−/− flies, two Parkinson's genes implicated in autophagic elimination (mitophagy) of mitochondria that have lost membrane potential [66–69]. Further, in *Rho-7*−/− flies, PINK1, which is typically imported and cleaved in the mitochondrion, is found almost exclusively in its full-length form, suggesting a role of the rhomboid protease in this processing [68]. Consistent with this finding, *Rho-7* genetically interacts with drosophila homologs of *PINK1* and *Parkin*, in a pattern consistent with *Rho-7* acting upstream of *PINK1* [68]. Since a role of the mammalian rhomboid protease Parl in PINK1 cleavage has recently been established (see below), these data indicate functional conservation of animal PARL family members in mitochondria quality control. However, in this respect it should be noted that, unlike flies, mice lacking mitochondrial rhomboid protease activity do not show embryonic lethality and synaptic defects [40]. Such phenotypic differences might reflect the possibility that mammalian Parl and drosophila Rho-7 could have a different set of substrates and/or mechanisms of regulation. Indeed, structural differences exist between these two orthologous proteins: in insects, the N-terminal domain and the loop that connects the distal TMH of the protease (TMH-A) to the TMH-1 of its rhomboid protease domain (Fig. 2A) are different from those found in mammals [38]. However, in mammals and higher vertebrates these two domains show considerable conservation, thereby supporting the possibility that during vertebrate evolution the function of the mitochondrial rhomboid protease might have been recruited to regulate mitochondrial pathways and activities not present in the fruit fly.

8. Parl, the mammalian mitochondrial rhomboid

Parl was first identified in a yeast two-hybrid screening in which the bait protein was PS2-C-cas, the C-terminal moiety of Alzheimer's Presenilin-2 that is produced by caspase cleavage during apoptosis [70,71]. PS2-C-cas includes part of the large hydrophilic loop and the last 3 TMHs of PS2 (a.a. 330–448); it shares a high degree of homology to the C-terminus of Alzheimer's Presenilin-1, it is highly conserved in mammals, and has anti-apoptotic activity [71]. The name Parl, therefore, is the acronym of Presenilin-associated Rhomboid-like protein [72]. However, the interaction between Parl and the Presenilin proteins is artifactual, possibly due to the highly hydrophobic nature of these two polytopic membrane proteins: thus, to date there is no evidence linking in any way Parl to the Alzheimer's presenilins [18,40].

The human Parl protein (Uniprot: Q9H300) is a 379 amino acid-long polypeptide containing seven transmembrane helices, organized in a "1 + 6 structure" (Fig. 2A). The first, proximal TMH ($_{101}$PLFFTVGFTGCAFGSAAIWQ$_{120}$) is termed TMH-A. The rhomboid protease domain spans amino acids 178–353, and the 6 TMHs that compose it are termed TMH-1, -2,-3,-4,-5, and -6, to allow an easy comparison with the 6 TMHs that constitute the catalytic serine protease domain found in all other prokaryotic and eukaryotic rhomboids [18].

Parl is inserted in the inner membrane of the mitochondrion. In cell transfection-based studies, over-expressed Parl-Flag (C-term) appears to expose its N-terminus in the mitochondrial matrix and its tagged C-terminus in the intermembrane space (IMS) [73]. This topology positions the catalytic site of the protease in the matrix. However, such orientation is not in agreement with evidence indicating that products of its cleavage are in the IMS, thereby warranting further investigations on the topology of the protease in other model organisms.

Parl is the mammalian ortholog of the yeast mitochondrial rhomboid protease Pcp1, which regulates mitochondria fusion through proteolytic processing of Mgm1 (discussed above). Expression of Parl in yeast cells lacking Pcp1/Rbd1 activity rescues Mgm1 cleavage and mitochondria fusion [63]. The mammalian homolog of Mgm1 is Opa1, which participates in mitochondrial fusion in association with Mfn1 [74], a dynamin-related GTPase of the outer mitochondrial membrane. However, even though a small amount (4%) of Opa1 is processed by Parl, the function of the rhomboid protease in mammals does not appear to be that of promoting mitochondrial fusion *per se* because the architecture and fusion rate of mitochondria in *Parl*−/− MEFs is similar to that measured in wild-type cells [40].

Parl+/− mice do not show any obvious phenotype [40]. Instead, *Parl*−/− mice develop normally until the 4th postnatal week, after which they start displaying severe growth retardation, with loss of muscle mass and postural defects. The animals die within 8 to 12 weeks by progressive cachexia, possibly aggravated by feeding and breathing problems. These animals have atrophic thymus and spleen, with at least 90% reduction in weight of these organs due to systemic cell death. Consistent with this phenotype, cells lacking Parl proteolytic activity have been shown to be more susceptible to intrinsic pro-apoptotic stimuli; this sensitivity correlates with a reduced amount of a cleaved form of Opa1 that, ultimately, impacts on the rate of cytochrome c release [40,41]. Whether in some cancer cells Parl activity is upregulated is not known, although this may be a mechanism tumor cells develop to acquire insensitivity to intrinsic pro-apoptotic stimuli or resistance to anti-cancer drugs.

The role of Parl in apoptosis has been suggested to be mediated through an additional pathway. Here, the congenital neutropenia protein Hax1 (Uniprot O00165) [75,76] presents the serine protease Omi/HtrA2 to Parl, to facilitate the generation of an active form of Omi/HtrA2 that prevents the accumulation of active Bax on the mitochondrial outer-membrane, which is required for cytochrome c release to the cytosol [77]. However, this model has been contested [78] and in contradiction to numerous reports that localize Hax1 on the ER, as well as with a wealth of studies that assign to Hax1 a different function and binding partners [79–81]. Therefore, to date the only established role of Parl in apoptosis is that that is linked to the Opa1-mediated mechanism of *cristae* remodeling.

9. Proteolytic regulation of Parl activity

The N-terminal domain of Parl (spanning amino acids 1–100) undergoes two distinct processing, termed α- and β-cleavage [82] (Fig. 2A). The α-cleavage (Fig. 3.1) occurs at position $G_{52}\downarrow F_{53}$, it is constitutive, and removes the mitochondria targeting sequence that imports the protease in the IMM. This processing, which is likely

executed by one of the proteases that typically import proteins in the IMM and matrix, produces MAMP, a *ma*ture *m*itochondrial form of *P*arl with rhomboid protease activity (Fig. 3.1).

The β-cleavage (Fig. 3.3) occurs 25 amino acids downstream of the α-cleavage, at position S_{77}↓A_{78}. This processing of Parl produces two moieties (Fig. 3.3). The first is the short Pβ-peptide ($_{53}$FRKAPRKVEPRRSDPGTSGEAYKRS$_{77}$) which, after being liberated in the cytosol reaches the nucleus through its nuclear localization sequence (see underlined amino acids above) [82]. Here, the Pβ-peptide activates mitochondria biogenesis *via* the expression of PGC1β, SIRT1, NRF1, Opa1, Mfn1, and Mfn2 (Fig. 3.3 inset) [83]. The actual target and mechanism of activity of the Pβ-peptide remain unknown, but given its small size it might act as a ligand of nuclear transcription factors that regulates the expression of these genes.

The second moiety produced by the β-cleavage of Parl is PACT, for *Pa*rl *C-t*erminal fragment. PACT spans amino acids 78–379 of Parl and retains rhomboid protease activity. Its function is to fragment mitochondria, possibly through a block membranes fusion (Fig. 3.3 inset) [84]. Indeed, whereas the ectopic expression of wild-type of Parl is associated to a massive fragmentation of the mitochondrial network, that of a mutant form of Parl in which β-cleavage abolished (*e.g.* L_{79}E) does not impact on the morphological organization of the organelles (Fig. 2B). Noteworthy, ablating the generation of a functional Pβ-peptide, but not of PACT, still results in massive mitochondrial fragmentation, thereby indicating that PACT is the sole moiety generated by β-cleavage that is mechanistically coupled to mitochondria fragmentation [73].

Arresting mitochondria fusion has far reaching consequences for the cell [85]. It is not surprising, therefore, that the generation of PACT is placed under tight control by sophisticated mechanisms of regulation. First, β-cleavage requires a mechanism of proteolysis that depends on Parl rhomboid activity supplied *in trans*: in transfected cells, a catalytically dead Parl S_{277}G mutant is not subjected to β-cleavage unless wild-type Parl is co-expressed [82]. Converging evidence suggest that this processing on Parl N-terminus might be directly executed by another molecule of Parl (Fig. 3.3), thereby implying that Parl N-terminus can access the IMM-immersed catalytic site. Second, Parl β-cleavage is sensitive to amino acid substitutions. Like many other proteins whose activity depends on proteolytic activation (*e.g.* caspases), mutations in either residues P2, P1, P1′ or P2′ (R_{76}E, S_{77}E, A_{78}E and L_{79}E) of the cleavage site abolish β-cleavage [82]. Third, a mutation on P1 of the β-cleavage site, S_{77}N, abolishes β-cleavage and is linked to non-familial cases of Parkinson's disease [86]. Fourth, β-cleavage of Parl is blocked by phosphorylation of three residues located in close proximity to the β-cleavage site. Endogenous and transfected Parl are phosphorylated at residues Ser_{65}, Thr_{69}, and Ser_{70}; phosphomimetic (Asp) substitutions at these amino acids impair β-cleavage without, however, affecting Parl rhomboid activity *per se* [73]. Thus, a still uncharacterized kinase/phosphatase switch (Fig. 3.2) is likely to be an integral component of the mechanism of regulation of Parl β-cleavage *in vivo*. Fifth, β-cleavage occurs in a domain that is strictly conserved in placental and marsupial mammals. This indicates that during the ~100 million years of mammalian evolution the domain harboring the β-cleavage site must have been subjected to strong purifying selection, which can only be explained by functional constraints linked to its regulatory role [73].

Since β-cleavage blocks mitochondria fusion, mechanisms that release this block and restore steady-state mitochondrial dynamics must exist. We have recently shown that the activity of PACT and, possibly, that of MAMP, is eliminated by yet another cleavage event, on the large loop that connects the TMH-A and TMH-1 of the protease (Fig. 2A). This processing, termed γ-cleavage, disrupts the 1 + 6 structure that defines every mitochondrial rhomboid protease and generates a form of Parl that appears to be catalytically inactive and unstable (Fig. 3.4) [38]. *In vitro* studies suggest that the activation of γ-cleavage is mechanistically coupled to that of β-cleavage and mediated by the activity of a serine protease. The identity of this protease is not known but our data exclude a role of Parl and Omi/HtrA2 in this process (our unpublished observations).

Why is mitochondria fusion arrest linked to the activation of the mitochondrial biogenesis pathway mediated by the Pβ peptide [83]? Two non-alternative scenarios can be hypothesized (Fig. 4). In the first, the block of membrane fusion may reflect an underlying defect in the metabolic competence of the organelle in which Parl is cleaved. In such case, therefore, the damage supported by the organelle would be "the signal" that activates β-cleavage. As a result, by blocking its ability to fuse back into healthy mitochondria this "compromised organelle" would become "quarantined", providing an opportunity for the cell to survey its respiratory competence and repair it. Should the damage suffered by this organelle be too extensive, during this quarantine this mitochondrion would eventually lose its electrochemical potential. At this point the depolarized organelle would disorganize its *cristae via* the Oma1-mediated stress-induced processing of Opa1 [87,88] and be eliminated by mitophagy *via* the PINK1/Parkin pathway (see below) [89]. In this way, Parl β-cleavage would simultaneously initiate new mitochondrial biogenesis through the Pβ peptide [83], and clear the cell of "old" or metabolically unresponsive or damaged mitochondria *via* PACT-mediated fusion arrest. In the second scenario, the small mitochondria generated by β-cleavage could serve as the platform on which "growth" of mitochondrial mass *via* the known mitochondrial biogenesis pathways can occur (Fig. 4).

The role of β-cleavage in human diseases has now started to emerge. As mentioned above, a mutation on the β-cleavage site that abolishes the ability of Parl to be subjected to β-cleavage has been associated to two sporadic cases of Parkinsonism [86].

Further, there might be a link with type-2 diabetes (T2DM). Patients with this metabolic conditions and Pima Indians, a group of Native Americans with a genetic predisposition for T2DM, have less mitochondrial mass and reduced Parl expression, a phenotype that is consistent with reduced β-cleavage and Pβ peptide-mediated signaling, which can activate the expression of a plethora of genes implicated in regulating mitochondrial biogenesis and dynamics (PGC1β, SIRT1, NRF1, Opa1, Mfn1, and Mfn2) [83].

What triggers β-cleavage remains to be determined, although in HeLa cells a number of stimuli that are known to trigger mitochondria fragmentation *via* fission activation do not induce β-cleavage of endogenous or transfected Parl (our unpublished observations). However, in HEK293 cells, β-cleavage seems to be activated when cells become highly or over confluent. Our ongoing studies on a mouse mutant deficient in Parl β-cleavage will contribute to elucidate the function of such an intricate mitochondrial processing event that impacts on mitochondria biology and human health in a unique way.

10. Role of Parl in the PINK1 mitochondria quality control pathway

Parkinson's disease (PD) is one of the most common neurodegenerative disorders affecting aging populations and consists in the progressive loss of dopaminergic neurons in the *substantia nigra* [90]. Even though PD is a sporadic neurological disorder, familial forms of the disease exist: in such cases, affected patients carry mutations in one of the PARK genes. One of them is *PINK1* [91,92], a gene that encodes a serine/threonine kinase that is found in the mitochondria and cytosol. PINK1 serves to signal the presence of dysfunctional mitochondria. The mechanism centers around the mitochondria membrane potential-dependent proteolysis of full-length PINK1 (63 kDa) in a shorter 52 kDa form that is then eliminated. This processing of PINK1 maintains low levels of PINK1 on healthy (polarized) mitochondria. Loss of membrane potential inhibits PINK1 cleavage, leading to its accumulation on the cytosolic side of the outer mitochondrial membrane; this event, in turn, recruits the E3 ubiquitin ligase Parkin protein on the cytosolic face of the mitochondrion, which presides the mitophagic elimination of the damaged (depolarized) organelle *via* the ubiquitination of Mfn1 and other OMM proteins [93–95]. Recent studies have shown that the 52 kDa form of PINK1 requires, and is

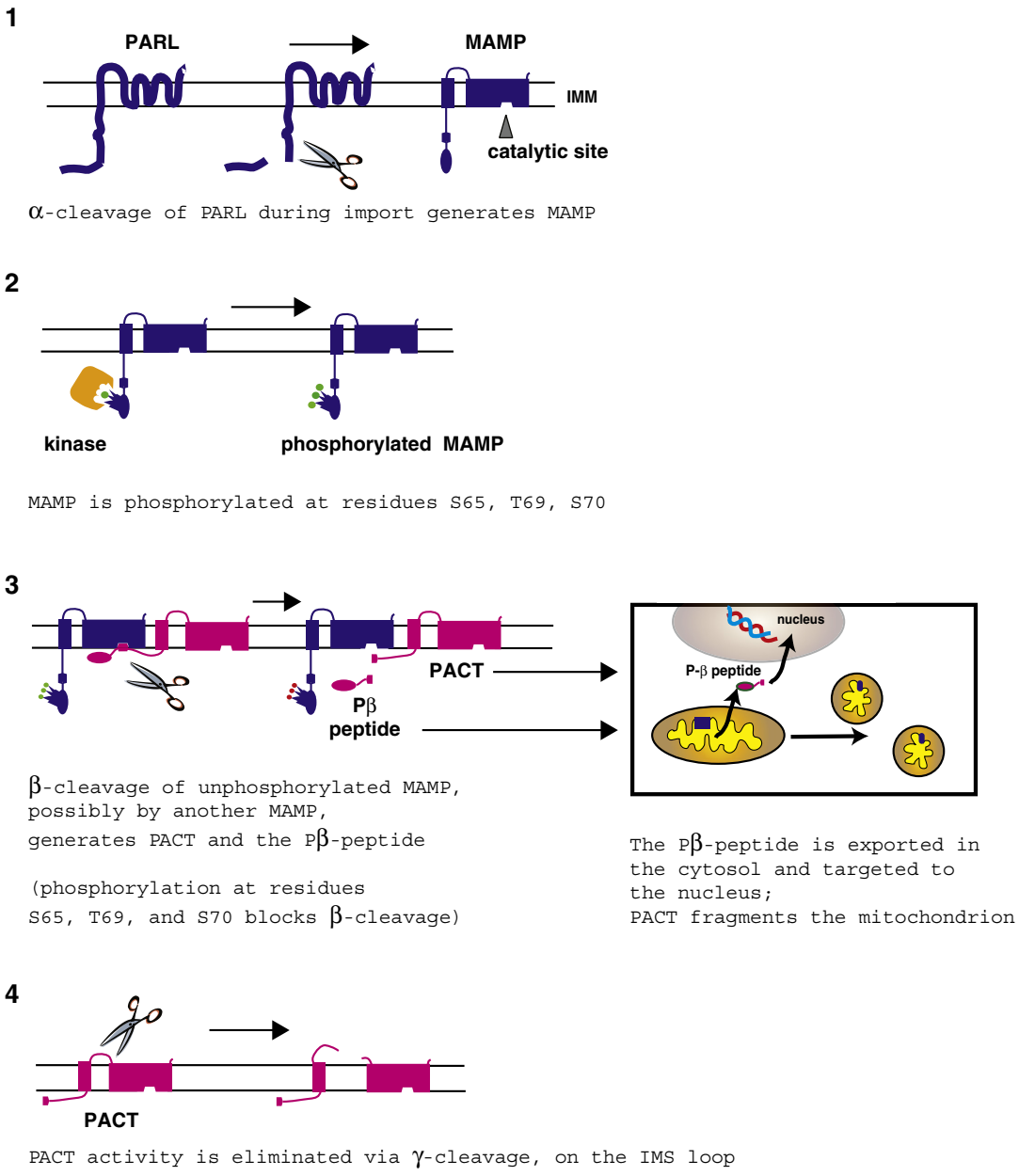


Fig. 3. Scheme showing the cascade of events regulating Parl activity in the mitochondrion. 1) Import and insertion of Parl in the inner (IMM) mitochondrial membrane. α-cleavage at amino acid 52↓53 removes the mitochondrial targeting sequence from Parl N-terminus [82]; the α-cleaved form of Parl is called MAMP, for mature mitochondrial Parl [82]. 2) MAMP N-terminus becomes constitutively phosphorylated by a still uncharacterized kinase (in yellow) at Ser-65, Thr-69, and Ser-70 [73]; note that the phosphorylation state of these residues has an impact on β-cleavage (phosphorylation blocks it), not on the rhomboid activity of the proteases *per se* [73]. 3) Dephosphorylated MAMP (in pink) is cleaved at amino acid 77↓78, most likely by another form of Parl (MAMP/PACT), *in trans* (in blue) [82]. β-cleavage generates two moieties: the N-terminal Pβ peptide (25 amino acid long), and PACT (Parl C-terminal fragment), which is an active rhomboid protease [73,82]. In the inset: β-cleavage generates the Pβ peptide, which is then exported to the nucleus [82] and fragments the mitochondrion [73]. 4) PACT activity is eliminated *via* γ-cleavage, on the loop-A of the protease [38].

probably directly generated by, the mitochondrial rhomboid protease Parl [86,89,96,97].

Another line of evidence links Parl to PD. A recent study has associated a missense mutation in Parl, $S_{77}N$, to two cases of sporadic parkinsonism [86]. This variant impairs the β-cleavage (see above) and, consequently the generation of PACT and of the Pβ-peptide, without, however, affecting Parl rhomboid protease activity *per se* [73,82,86] (Figs. 3 and 4). Interestingly, similar to a catalytically dead Parl $S_{277}G$ mutant, the $S_{77}N$ mutation displays inefficient Parkin recruitment to mitochondria that have lost membrane potential. Hence, the $S_{77}N$ mutation seems to impact on mitochondria quality control independent of PINK1 processing, for which Parl rhomboid activity is needed. Future studies will likely clarify the implication of Parl β-cleavage in parkinsonism and mitochondria quality control.

11. Rhomboids substrate recognition and specificity

Typically, proteases use residues flanking the cleavage site of the substrate to recognize the scissile bond to cleave. This paradigm seems to apply also to bacterial and RHO rhomboids (but not to other types of I-CLiPs); here, only amino acids harboring a small side chain are permitted at the P1 position (A,G,C,S), whereas large, hydrophobic side chains are required at P4 position (I,M,YF,W,LV) and preferred at the P2′ (F,IM,V,A,CL,T,W). Instead, some residues are not tolerated at

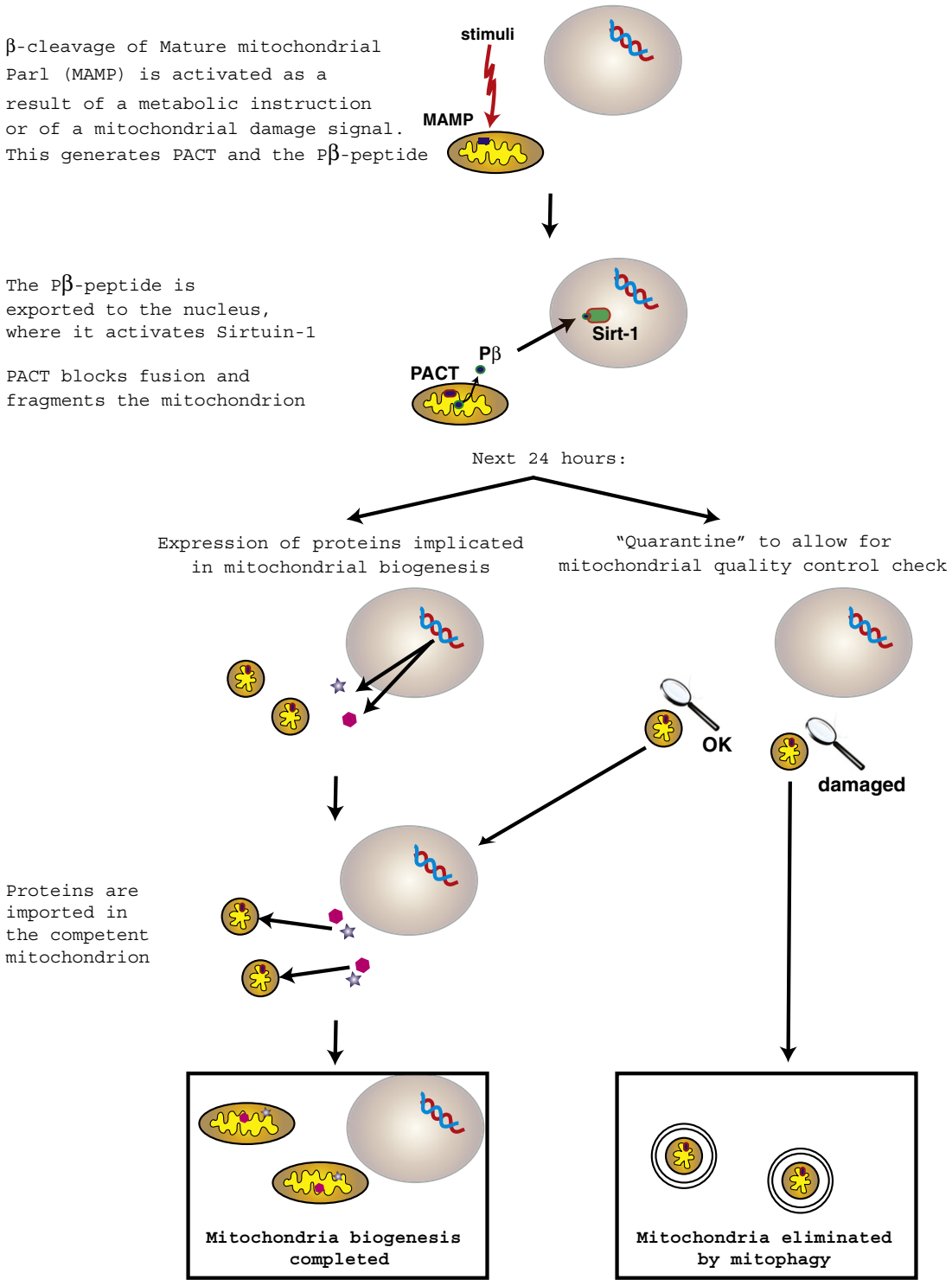


Fig. 4. Models explaining the possible function of Parl β-cleavage, which couples mitochondria dynamics [73] to mitochondria biogenesis [83] *via* the generation of PACT and of the nuclear Pβ peptide, respectively.

position P5 (W,D), P3 (W,P,D), P2 (W,F), P1′ (P,D), and, possibly, P2′ (GYHKRDENQP) [5,98]. For mitochondrial rhomboids less is known. However, the β-cleavage site of Parl, whose cleavage is strictly dependent of Parl activity supplied in trans [82], is consistent with these requirements ($A_{P5}Y_{P4}K_{P3}R_{P2}S_{P1}{\downarrow}A_{P1}'L_{P2}'$), thereby further supporting the possibility that this cleavage is indeed directly executed by Parl. As for the cleavage site of PINK1 and Mgm1, which depend on mitochondrial rhomboid activity, the residue at position P1 (for Mgm1) and P2′ (for PINK1) do not seem to fit with this consensus sequence (Mgm1 — site1: $G_{P5}T_{P4}V_{P3}P_{P2}T_{P1}{\downarrow}A_{P1}'T_{P2}'$; Mgm1 — site 2: V_{P5}, $P_{P4}T_{P3}A_{P2}T_{P1}{\downarrow}L_{P1}'I_{P2}'$; PINK1: $A_{P5}V_{P4}F_{P3}L_{P2}A_{P1}{\downarrow}F_{P1}'G_{P2}'$;) [53,96].

Current evidence [5,99] suggest a model where the recruitment of a rhomboid substrate occurs in two phases. The first step involves the

interaction of the substrate's TMH to a membrane-immersed rhomboid exosite. The second step consists of the recognition of residues encompassing the cleavage site of the substrate, and their docking into the active site cleft of the protease. Accordingly, a substrate's cleavage sites can reside considerably distant from its TMH, as it is the case for Parl β-cleavage site, which is located 24 amino acid residues away from TMH-A. It is tempting to speculate that TMH-A, in PARL family members, and TMH-B, in RHO family members, could participate to the formation of the exosite(s) that govern the substrate specificity of the eukaryotic rhomboids. This possibility is supported by the fact that, in the mammalian mitochondrial rhomboid Parl protein, TMH-A harbors a conserved GxxxG TMHs-dimerization motif [100] ($_{101}$PLFFTVGFTGCAFGSAAIW$_{118}$).

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